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Award Number DAMD17-89-C-9050

TITLE: A Medical Research and Evaluation Facility (MREF) and Studies Supporting the Medical Chemical Defense Program

SUBTITLE: Task 97-47: Analysis of NF-KB in Pulmonary Tissue From Laboratory Rodents Exposed to HD

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REPORT DATE: December 1999

TYPE OF REPORT: Final (Task 97-47)

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1999	Final

4. TITLE AND SUBTITLE
A Medical Research and Evaluation Facility (MREF) and Studies Supporting the Medical Chemical Defense Program

SUBTITLE: Task 97-47: Analysis of NF-KB in Pulmonary Tissue From Laboratory Rodents Exposed to HD

5. FUNDING NUMBERS
DAMD17-89-C-9050

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS
HD, Pulmonary Tissue

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Executive Summary

Vesicants, such as sulfur mustard (HD), have several routes of exposure, including dermal, ocular, and pulmonary. The most hazardous effects of HD are manifested in the respiratory tract following inhalation of HD vapor. Acute lung edema will result when the pulmonary system is exposed to sufficiently high levels of HD (Eisenmenger *et al.*, 1991). One research objective of the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD) is to assess the risk of pulmonary HD exposure and to identify early markers of exposure. HD is a highly reactive compound that is unstable in biological fluids, making it difficult to quantify the dose received deep in the lungs after an aerosol exposure. Previous investigators found that, following an inhalation exposure, clinical manifestations of HD-induced injury were found primarily in the upper airway passages (Papirmeister *et al.*, 1991). Literature references indicate that intravenous and subcutaneous HD exposures of rodents can be used to study pulmonary toxicity, thus circumventing the need for generating HD vapor for inhalation (Maisonneuve *et al.*; 1993 Maisonneuve *et al.*, 1994; Elsayed *et al.*, 1992). Data in the literature also demonstrate that subcutaneous injection of the monofunctional HD analog, butyl 2-chloroethyl sulfide, induces free radical-mediated oxidative pulmonary damage in mice (Elsayed *et al.*, 1992). As part of a USAMRICD Study, Protocol No. 1-04-97-000-A-741, lung specimens from rats that had been intravenously exposed to HD were sent to Battelle's Medical Research and Evaluation Facility (MREF) and assessed for activation of nuclear factor *kappa* B (NF-*k*B), a transcription factor involved in the inflammatory response.

NF-*k*B is a ubiquitous transcription factor of particular importance in the early amplification of the inflammatory response. NF-*k*B is normally present in the cell as inactive cytosolic complexes of NF-*k*B and the inhibitory protein called I-*k*B. Extracellular signals target the NF-*k*B:I-*k*B complexes, leading to a phosphorylation-dependent proteolytic degradation of I-*k*B. Activated NF-*k*B is released to translocate to the nucleus where it can bind to the promotor region of genes encoding various inflammatory mediators (e.g., chemokines and cytokines). To study the relationship between NF-*k*B transcription factors and oxidative tissue damage, studies were conducted under Task 97-47 to assay activated NF-*k*B in pulmonary tissue from animals following intravenous exposure to HD under time- and dose-dependent experimental circumstances.

An Electrophoretic Mobility Shift Assay (EMSA) was used to detect the presence of deoxyribonucleic acid (DNA) binding proteins by their ability to retard the migration of DNA oligonucleotide probes during electrophoresis. Protein samples were first extracted from pulmonary tissue, then incubated with a radiolabeled NF-*k*B oligonucleotide probe containing the putative binding site recognized by NF-*k*B. Electrophoretic mobility retardation of the protein:NF-*k*B oligonucleotide probe complex relative to uncomplexed NF-*k*B oligonuclotide probe was visualized on developed x-ray film that had been exposed to the gel. Densitometric quantitation was then performed to assess the effects of HD exposure on NF-*k*B activation. A nuclear protein extract from HeLa cells known to contain activated NF-*k*B was evaluated concurrently with study samples as an experimental control. Unlabeled DNA oligonucleotide probes, either non-specific or specific for NF-*k*B binding proteins, were also evaluated to demonstrate response specificity.

NF-*k*B binding proteins in HD-treated samples were not detected at a level significantly above that found to be present in control samples. Statistical analysis did show a small but significant reduction in NF-*k*B binding proteins in samples taken from animals exposed to 3 mg/kg and 6 mg/kg HD and sacrificed at 24 hr following exposure. These alterations are minor in magnitude, and are consistent with the delayed onset of toxicity characteristic of HD. When expressed as a fraction of vehicle control, the average levels of NF-*k*B in samples taken at 24 hr following exposure were 0.93, 0.81, and 0.77 for the 1 mg HD/kg, 3 mg HD/kg, and 6 mg HD/kg dose groups, respectively. These data suggest that alterations in the level of NF-*k*B does not mediate HD-induced pulmonary inflammation in rodents intravenously exposed to HD.

TASK 97-47

ANALYSIS OF NF-*k*B IN PULMONARY TISSUE FROM LABORATORY RODENTS EXPOSED TO HD

1.0 INTRODUCTION

Sulfur mustard [(bis-(2-chloroethyl)sulfide; HD)] is a lipid soluble, bifunctional alkylating agent that is preferentially cytotoxic to cells with a high mitotic index. Principal risks of HD exposure include percutaneous and inhalation routes. Large, fluid-filled blisters, characterized by dermal/epidermal separation, occur in humans following percutaneous exposure. Inhalation exposure causes toxicity to bronchial epithelial cells that can, if severe enough, lead to bronchial congestion and death (Eisenmenger *et al.*, 1991).

Although the exact mechanism of HD-induced tissue damage is not fully understood, HD does cause a marked inflammatory effect. Following dermal exposure of mice, gene expression of a number of pro-inflammatory mediators is elevated in HD-exposed skin. Mediators that are upregulated include interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), macrophage inflammatory protein-2 (MIP-2), and acute phase reactive proteins (Sabourin and Casillas, 1998; Casillas *et al.*, 1997; Blank *et al.*, 1998; Danne *et al.*, 1999).

Acute pulmonary inflammation involves the initiation of an inflammatory cascade leading to an influx of cellular mediators that increase vascular permeability and lead to additional damage through the release of reactive oxygen species (ROS), proteases, and additional inflammatory mediators. ROS have been shown to initiate an inflammatory cascade through NF-*k*B activation (Schreck *et al.*, 1991; Blackwell *et al.*, 1996). Since the effects of acute pulmonary inflammation are in part initiated through ROS, inflammation following exposure to HD may also be mediated through NF-*k*B. The purpose of this study was to analyze NF-*k*B levels in nuclear extracts of pulmonary tissue from rats that had been intravenously exposed to HD.

2.0 MATERIALS AND METHODS

Adenosine 5'-triphosphate [γ-^{32}P] used for labeling oligonucleotide probes, was purchased from NEN-Dupont Life Sciences Products (Boston, MA). T4 polynucleotide kinase enzyme, oligonucleotides corresponding to the transcription factors AP-1, AP-2, SP-1 and NF-κB, and HeLa Nuclear Extract used in control reactions were purchased from Promega Corporation (Madison, WI). Poly (dI-dC)•Poly (dI-dC), used as a non-specific competitor, was purchased from Pharmacia Biotech (Piscataway, NJ). Dounce homogenizers were purchased from Daigger Company (Lincolnshire, IL). G-25 Sephadex spin columns, used to purify radioactively labeled probes, were purchased from Worthington Biochemical Company (Freehold, NJ). BSA protein standards and bicinchoninic acid (BCA) assay reagents for measuring protein content were purchased from Pierce Chemical Company (Rockford, IL). Mini-gel electrophoresis equipment, GS-700 densitometer, Multi Analyst™ software, vacuum gel drying apparatus, cellophane sheets, filter paper, tris-borate-EDTA buffer, bromophenol blue dye, and polyacrylamide gels were purchased from BioRad (Hercules, CA). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The scintillation counter, model LS-3801, was manufactured by Beckman Coulter Corporation (Fullerton, CA).

2.1 Animal Model

The animal model used for these studies was the male Sprague Dawley rat (n = 65), weighing approximately 240 - 270 gm. All animal husbandry and manipulations were performed at USAMRICD under USAMRICD Protocol No. 1-04-97-000-A-741.

2.2 HD Exposure

At USAMRICD, experimental animals were euthanatized at approximately 3 hr, 6 hr, and 24 hr following intravenous HD exposure at doses of 0 (isopropanol vehicle control and naive),

1, 3, or 6 mg HD/kg of body weight. The left pulmonary lobe was dissected, rinsed in normal saline, snap-frozen, and then sent to the MREF on dry ice for NF-*k*B analyses.

2.3 Tissue Preparations

Left lung samples harvested from rats that had been exposed intravenously to HD were sent to the MREF on dry ice. Samples were cataloged prior to being stored at –70 C. When removed from the freezer for processing, samples were maintained in liquid nitrogen. Nuclear proteins were isolated from tissue samples following MREF Method No. 53/*In Vitro* (Appendix B). Using a calibrated analytical balance, individual lung samples were weighed and the data recorded. Samples were placed into a liquid nitrogen-cooled mortar and reduced to a fine powder with a liquid nitrogen-cooled pestle. Next, samples were transferred to a Dounce homogenizer that contained a homogenization buffer with protease inhibitors (dithiothreitol and phenylmethylsulfonyl fluoride). After incubation in the first homogenization buffer, samples were "Dounced" three times with a loose-fitting plunger, followed by five times with a tight-fitting plunger, to release the nuclei. The tissue suspensions were briefly centrifuged to pellet cell debris. Supernatants were transferred into a clean centrifuge tube and centrifuged at 3,600 x g for 15 min at approximately 4 C. The supernatants were discarded and any remaining droplets were removed from the sides of the tube. Next, the nuclei-containing pellets were suspended in a high-salt homogenization buffer containing protease inhibitors (dithiothreitol, phenylmethyl-sulfonyl fluoride, aprotinin, pepstatin, and leupeptin) to extract the nuclear proteins. The samples were incubated on ice for approximately 30 min with intermittent vortexing. After centrifugation at 17,900 x g for 5 min at 4 C, each supernatant was combined with three volumes of a high-glycerol, low-salt buffer containing protease inhibitors (dithiothreitol, phenylmethyl-sulfonyl fluoride, aprotinin, pepstatin, and leupeptin) for storage at –70 C.

2.4 Protein Determinations

The concentration of nuclear protein extracted from each tissue sample was determined using the BCA Method standardized with a commercial preparation of bovine serum albumin

(BSA). Protein samples were run in duplicate and the results averaged. Calculations based on the protein concentration results were used to determine the volume of protein sample required in each Electrophoretic Mobility Shift Assay (EMSA) reaction.

2.5 Preparation of the NF-*k*B Oligonucleotide Probe

A double-stranded synthetic oligonucleotide containing a binding site specific for NF-*k*B was ^{32}P-labeled to high specific activity using T4 polynucleotide kinase, which catalyzes the transfer of a labeled γ-phosphate from ATP to the 5'-hydroxyl group of the NF-*k*B oligonucleotide. Unincorporated nucleotides were removed after the labeling reaction by spin-column chromatography using Sephadex resin. The labeled probe was then quantified by Cerenkov counting with a scintillation counter, and stored at approximately –20 C for less than one week before being used in an EMSA.

2.6 EMSA Binding Reactions

Characterization of sample proteins involved a series of developmental experiments designed to test the EMSA under several different conditions. Binding reactions were performed in a buffer containing nuclear extract, labeled NF-*k*B probe, cationic salts, reducing agents, glycerol, and for control reactions, non-specific or specific competitor oligonucleotides. Parameters that were optimized included binding buffer components, protein extraction buffer components, binding reaction time and temperatures, and probe labeling methods.

Positive control reactions using commercially produced HeLa nuclear extract, known to bind to NF-*k*B, were used to demonstrate satisfactory performance of the optimized system. Additionally, the specificity of the nuclear extract for the NF-*k*B oligonucleotide probe was demonstrated by performing a competition reaction in which an excess of unlabeled NF-*k*B oligonucleotide was added to a reaction containing HeLa nuclear extract and labeled NF-*k*B probe. A decrease in the signal intensity of the shifted band measured densitometrically from the autoradiograph was evidence of the specificity and affinity of the labeled probe for the binding proteins of interest.

Analyses of protein-DNA interactions were performed according to MREF Method No. 53/*In Vitro* (See Appendix B). Briefly, 10-20 µg of sample protein was incubated in binding buffer at room temperature for approximately 20 min in the presence of poly (dI-dC)•poly (dI-dC), which competes with proteins in the sample extracts that might otherwise bind nonspecifically to the labeled NF-*k*B probe. Labeled NF-*k*B probe was added and the reactions were allowed to proceed on ice for an additional 15 min. A loading buffer containing bromophenol blue dye was added to each reaction tube before loading contents onto a polyacrylamide gel cassette for electrophoresis at approximately 140 volts, constant voltage, at room temperature.

After the dye front had traveled approximately 80 percent of the distance down the vertical face of the gel, electrophoresis was stopped. The electrophoresis gel was released from the cassette and sandwiched between a piece of filter paper and a piece of cellulose film, then vacuum-dried at approximately 80 C for 2 hr. Dried gels were exposed to X-ray film at approximately –20 C in an exposure cassette using an intensifying screen. The exposure length (13 hrs) was equivalent for autoradiographs used for densitometric comparisons. X-ray films were developed with an automated processor.

2.7 Data Analysis

NF-*k*B consists of protein dimers that typically produce two sequence-specific gel shifted complexes, a p50/p50 homodimer and a p50/p65 heterodimer, that are visible on an autoradiograph. Autoradiographs were scanned using a Bio-Rad GS-700 densitometer and Multi Analyst software. For each sample, the volume densities, expressed as the optical density x mm^2, for the two complexes were added. When vehicle control samples were evaluated under identical experimental conditions concurrently with samples from HD-exposed animals, data were standardized and expressed as a fraction of vehicle control response. The fractional response was calculated by dividing the densitometry value for the HD-exposed samples by the average value for the vehicle control samples at the same time point.

A two-factor analysis of variance (ANOVA) model was fitted to the NF-*k*B fractional response data to determine the effects of HD dose and time. Model results indicated that neither

the HD dose, nor the interaction between HD dose and time, produced statistically significant effects. Thus, a one-factor ANOVA model was fitted to examine the effect of time. In addition, a second one-factor ANOVA model that treated each combination of HD dose and time separately, was performed.

In both cases, model parameters were used to estimate the mean NF-*k*B fractional response and standard error. A t-test was used to determine whether the mean NF-*k*B fractional response differed from one, which would indicate that the response for HD-dosed animals was significantly different from that of control animals. A Bonferroni adjustment for multiple comparisons (Neter *et al.*, 1989) was applied when evaluating these estimates. In addition, Tukey's multiple comparisons procedure was used to compare mean ratios between groups. Both the Bonferroni adjustment and Tukey's test control the experimentwise error rate at the 0.05 level.

3.0 RESULTS

Statistical analyses were performed using numerical values obtained from densitometric data (as described in section 2.7 Data Analysis) to determine whether HD was capable of stimulating NF-*k*B binding proteins. Overall results indicate that NF-*k*B binding protein activity was not increased in rat lung tissues from animals euthanatized 3 hr, 6 hr, or 24 hr following i.v. exposure to 1, 3, or 6 mg HD/kg body weight (Figures 1 through 9). Statistical analyses did indicate a minor reduction in NF-*k*B binding proteins in samples taken from animals exposed to 3 and 6 mg HD/kg body weight and euthanatized 24 hr following exposure (Figures 8 and 9).

Adjusting the components and pH of the tissue homogenization buffers optimized nuclear extract isolation conditions. Additionally, experimental conditions of the binding reaction were adjusted to achieve the optimal titer of probe, concentration of nuclear extract, incubation time and temperature, and concentration of nonspecific competitor. Positive control reactions using commercially produced HeLa nuclear extracts, known to bind to NF-*k*B, were used to demonstrate satisfactory performance of the optimized system. (Figures 10 through 12).

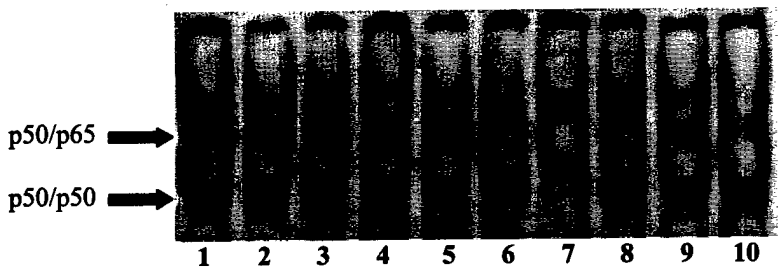

Figure 1. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 1 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

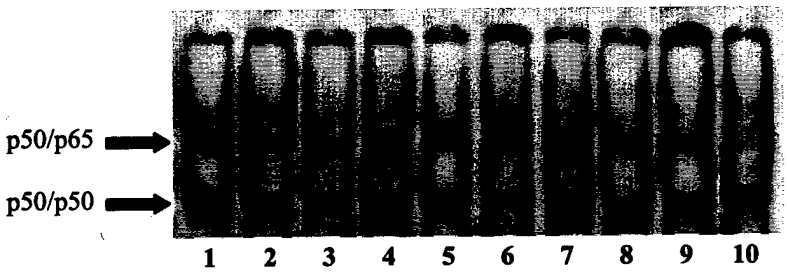

Figure 2. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 3 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

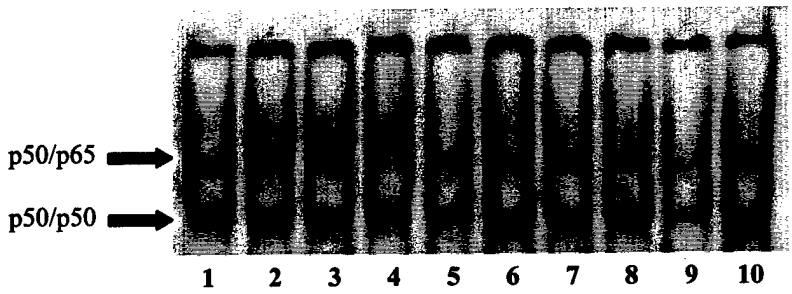

Figure 3. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 6 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

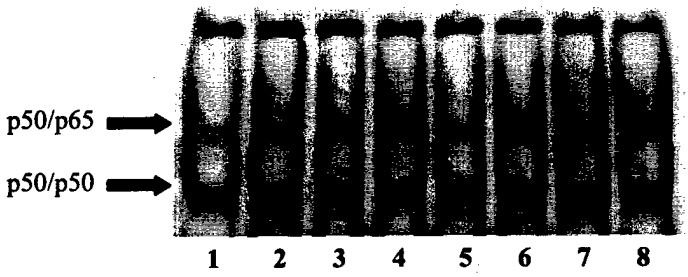

Figure 4. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-4) and 1 mg/kg HD (Lanes 5-8) exposed samples. Animals were euthanatized 6 hr following exposure.

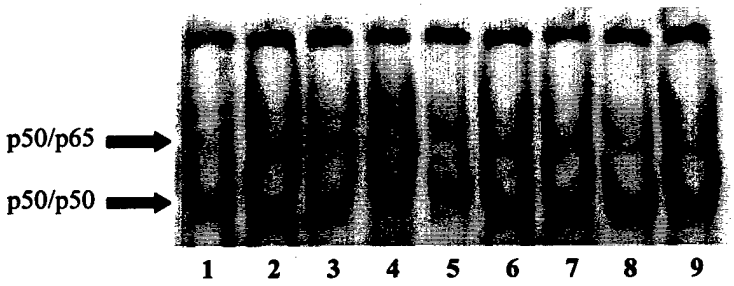

Figure 5. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-4) and 3 mg/kg HD (Lanes 5-9) exposed samples. Animals were euthanatized 6 hr following exposure.

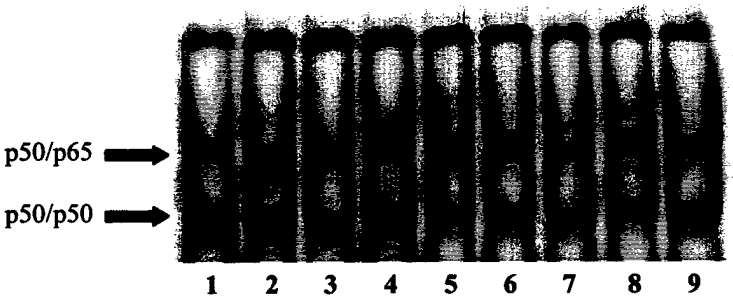

Figure 6. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-4) and 6 mg/kg HD (Lanes 5-9) exposed samples. Animals were euthanatized 6 hr following exposure.

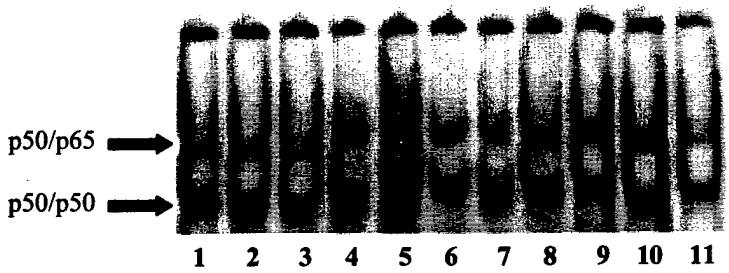

Figure 7. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 1 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.

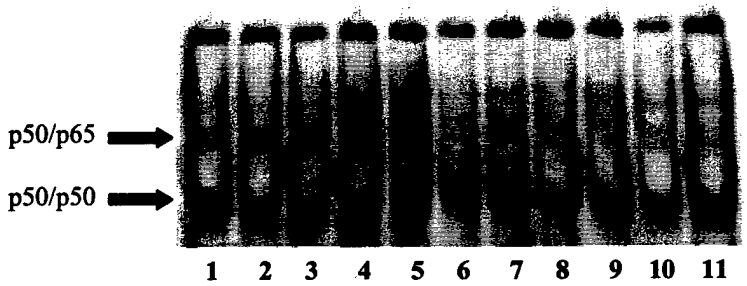

Figure 8. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 3 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.

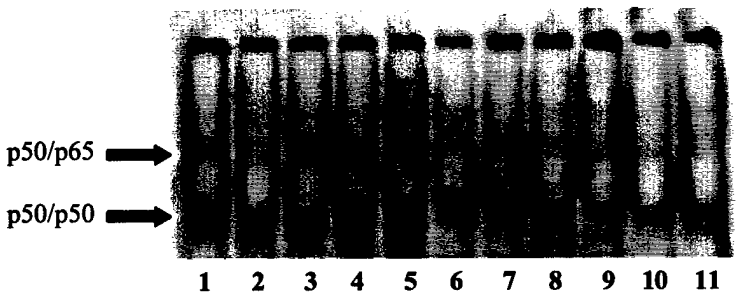

Figure 9. Autoradiograph of NF-*k*B shifted complexes for IPA (Lanes 1-5) and 6 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.

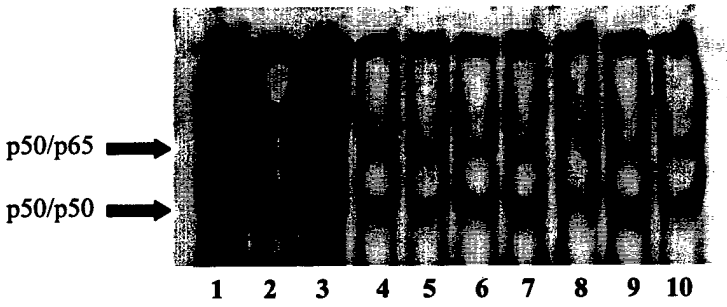

Figure 10. Autoradiograph of NF-*k*B shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-*k*B oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3), IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9-10) exposed samples. Animals were euthanatized 3 hr following exposure.

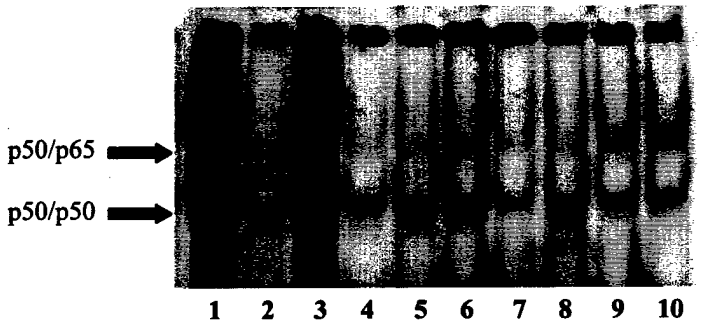

Figure 11. Autoradiograph of NF-*k*B shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-*k*B oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3, IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9-10) exposed samples. Animals were euthanatized 6 hr following exposure.

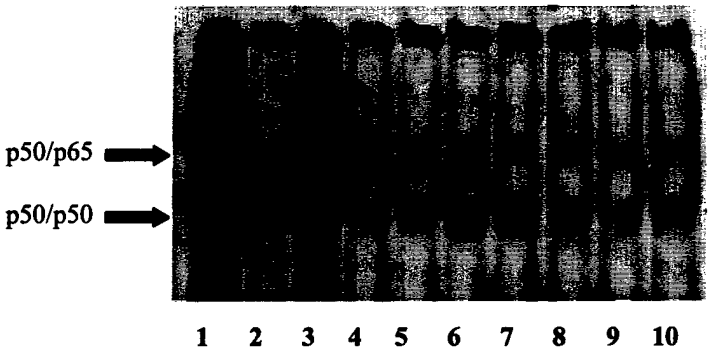

Figure 12. Autoradiograph of NF-*k*B shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-*k*B oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3), IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9-10) exposed samples. Animals were euthanatized 24 hr following exposure.

4.0 DISCUSSION

Studies were conducted to investigate the premise that the redox sensitive transcription factor Nuclear Factor-*kappa* B (NF-*k*B) is involved in mediating the inflammatory response that occurs after exposure to sulfur mustard. Most genes contain promotors or enhancers within their regulatory elements, which contain binding sites for multiple transcription factors, including NF-*k*B. NF-*k*B is normally present in the cytosol in a non-DNA-binding form. Extracellular signals lead to its translocation into the nucleus where it can bind to gene promoter and/or enhancer sequences and activate the expression of genes involved in inflammatory and immune responses (Baeuerle and Baltimore, 1988; Schreck *et al.*, 1991). Acute pulmonary inflammation, such as is caused by inhalation of HD, involves the stimulation or injury of cells that line the respiratory tract. These cell types may initiate an inflammatory response through the release of mediators that increase vascular permeability and attract blood-borne inflammatory cells. The edematous response leads to reduced pulmonary function. The influx of inflammatory cells into the exposed tissue may produce additional damage through the release of reactive oxygen species, proteases, and other inflammatory mediators (Gordon and Amdur,

1991). There is evidence that vesicant-induced pulmonary toxicity in rodents is mediated via oxidative stress mechanisms. Elevations in antioxidant enzyme activities, lipid peroxidation, and oxidized glutathione – all known inducers of free radical-mediated oxidative stress – have been reported (Elsayed *et al.*, 1992).

The Electrophoretic Mobility Shift Assay (EMSA) was optimized for use as a qualitative measure of the presence of NF-*k*B. EMSA is a technique designed to detect the presence of DNA binding proteins by their ability to retard the migration of DNA fragments during electrophoresis. A total of 85 samples were evaluated for the development of methods used in protein extraction and quantification, and for performing EMSA on submitted tissue. (Table 3). Results indicate that, at the concentrations of HD and time points evaluated in Task Order 97-47, alterations in the level of NF-*k*B do not mediate HD-induced pulmonary inflammation in rodents intravenously exposed to HD. Neither the interaction between HD dose and time, nor the HD dose effect was shown to be statistically significant.

Even though NF-*k*B was not found to be predictive of HD-induced pulmonary damage in studies conducted under this task, the literature does indicate that activated NF-*k*B may be predictive of toxicant-induced pulmonary damage (Blackwell *et al.*, 1996). Studies suggest a role for NF-*k*B in the generation of acute inflammatory responses and further suggest that NF-*k*B may be a potential avenue for anti-inflammatory therapy (Motoyoshi *et al.*, 1996; Blackwell *et al.*, 1996). If activation of transcription factors, such as NF-*k*B, is an event associated with vesicating doses of HD, then the biochemical events leading to activation may provide insight into the development of new therapeutic strategies to minimize HD-induced pulmonary toxicity.

5.0 ACKNOWLEDGEMENTS

This report reflects the collaborative effort of Dr. Alfred Sciuto (USAMRICD), Dr. James Blank, Ms. Michele Danne, and the technical efforts and dedication of Ms. Kristi Buxton. The statistical support was provided by Ms. Nancy Niemuth and Ms. Jennifer Holdcraft. The quality assurance support was provided by Ms. Elisha Morrison and Ms. Jessica Evans. The secretarial support was provided by Ms. Charlotte Hirst, Ms. Bonnie Snodgrass, and Ms. Katie Wiseman. Dr. Carl Olson provided invaluable editorial comments.

Table 1. Model Estimated Mean, Standard Error, and p-Value for Difference from One for NF-*k*B Fraction of Vehicle Control Over Three HD-Doses at Each Time

Time (hr)	NF-*k*B Fraction of Vehicle Control	Standard Error	p-Value
3	0.91	0.035	0.018
6	0.98	0.036	0.510
24	0.84	0.032	<0.001*

* Group mean is significantly different from one. A Bonferroni adjustment for multiple comparisons was applied, so that p-values were compared to 0.017 to maintain an overall 0.05 level of significance.

Table 2. Model Estimated Mean, Standard Error, and p-Value for Difference from One for NF-*k*B Fraction of Vehicle Control for Each HD Dose Group and Time

Time (hr)	HD Dose (mg/kg)	NF-*k*B Fraction of Vehicle Control	Standard Error	p-Value
3	1	0.91	0.058	0.129
3	3	0.89	0.058	0.072
3	6	0.94	0.058	0.332
6	1	1.05	0.065	0.444
6	3	0.90	0.058	0.084
6	6	1.00	0.058	0.949
24	1	0.93	0.053	0.229
24	3	0.81	0.053	0.001*
24	6	0.77	0.053	<0.001*

* Group mean is significantly different from one. A Bonferroni adjustment for multiple comparisons was applied, so that p-values were compared to 0.006 to maintain an overall 0.05 level of significance.

Table 3. Summary of Lung Tissue Samples Tested

Assigned ID Number	Treatment Groups	Timepoint (hr)
ICD R32 A729*	CG	.75 - 1
ICD R1 A729*	CG	.75 - 1
ICD R18 A729*	AIR	.75 - 1
ICD R9 A729*	AIR	.75 - 1
mlung #1*	CG	1
mlung #2*	CG	4
mlung #3*	CG	8
mlung #4*	CG	12
mlung #5*	CG	24
mlung #6*	AIR	1
mlung #7*	AIR	4
mlung #8*	AIR	8
mlung #9*	AIR	12
mlung #10*	AIR	24
729 R43*	CG	.75 – 1
729 R38*	AIR	.75 – 1
729 R37*	CG	.75 – 1
729 R39*	AIR	.75 – 1
729 R26*	AIR	.75 – 1
729 R33*	CG	.75 - 1
21	IPA	6
26	IPA	6
25	IPA	6
32	IPA	6
30	1 mg/kg HD	6
31	1 mg/kg HD	6
24	1 mg/kg HD	6
29	1 mg/kg HD	6
15	3 mg/kg HD	6
18	3 mg/kg HD	6
23	3 mg/kg HD	6
22	3 mg/kg HD	6
17	3 mg/kg HD	6
20	6 mg/kg HD	6
19	6 mg/kg HD	6
27	6 mg/kg HD	6
16	6 mg/kg HD	6
28	6 mg/kg HD	6
33	IPA	3
35	IPA	3

Table 3. (Continued)

Assigned ID Number	Treatment Groups	Timepoint (hr)
39	IPA	3
42	IPA	3
46	IPA	3
52	Saline	3
54	Saline	3
55	Saline	3
56	Saline	3
36	1 mg/kg HD	3
40	1 mg/kg HD	3
43	1 mg/kg HD	3
47	1 mg/kg HD	3
50	1 mg/kg HD	3
34	3 mg/kg HD	3
37	3 mg/kg HD	3
41	3 mg/kg HD	3
44	3 mg/kg HD	3
48	3 mg/kg HD	3
38	6 mg/kg HD	3
45	6 mg/kg HD	3
49	6 mg/kg HD	3
51	6 mg/kg HD	3
53	6 mg/kg HD	3
60	IPA	24
66	IPA	24
68	IPA	24
72	IPA	24
77	IPA	24
57	1 mg/kg HD	24
61	1 mg/kg HD	24
64	1 mg/kg HD	24
67	1 mg/kg HD	24
73	1 mg/kg HD	24
76	1 mg/kg HD	24
58	3 mg/kg HD	24
62	3 mg/kg HD	24
69	3 mg/kg HD	24
70	3 mg/kg HD	24
74	3 mg/kg HD	24
78	3 mg/kg HD	24
59	6 mg/kg HD	24

Table 3. (Continued)

Assigned ID Number	Treatment Groups	Timepoint (hr)
63	6 mg/kg HD	24
65	6 mg/kg HD	24
71	6 mg/kg HD	24
75	6 mg/kg HD	24
79	6 mg/kg HD	24

CG = Phosgene
IPA = Isopropyl alcohol
HD = Sulfur mustard
* = Samples used for development of method

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APPENDIX A

Battelle SOP MREF. V-018-01

Manual Number 46

Battelle SOP Number: MREF. V-018-01

STANDARD OPERATING PROCEDURE (SOP) FOR THE MOLECULAR DEVICES THERMOmax MICROPLATE READER

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I. Scope

This SOP encompasses all technical staff who utilize the microplate reader

II. Purpose

This SOP describes the general operation, maintenance and calibration of the Molecular Devices THERMOmax Microplate Reader.

III. References

A. Battelle SOP Number: MREF. XI-008, SOP for use of Equipment and Logbooks

B. Battelle SOP Number: MREF. XI-009, SOP for Recording, Reviewing and Correcting Raw Data

C. Molecular Devices THERMOmax User's Manual

D. Molecular Devices SOFTmax PRO Version 1.2 Manual

IV. Definitions

A. GLP-Good Laboratory Practice as defined in 21 CFR Part 58, 40 CFR Part 160 and 40 CFR Part 792.

B. The Molecular Devices THERMOmax MAXline Microplate Reader is a vertical pathlength photometer which measures absorbencies of the contents of the wells of microtiter plates commonly used for Enzyme-Linked Immunosorbent Assays (ELISAs) and spectrophotometric assays performed in small volumes.

V. Procedures

A. Prior to a specific application, check the availability of the appropriate filters needed for the particular experiment.

B. Start the computer with the SOFTmax PRO Program installed on the hard drive. Turn on the plate reader using the power switch located on the rear right hand side and allow it to warm up for approximately 15 minutes before use. The default protocol file will display the parameters (mode, wavelength, automatic mixing, run time, read interval) used to read the microplate.

Check that the date and time are accurate prior to collecting data.

C. Operate the plate reader using the SOFTmax PRO software in accordance with (IAW) the manufacturer's instructions. Alternatively, you may operate it independent of the software by using the membrane key pad on the top of the reader in conjunction with a printer connected through its parallel port.

D. Using the keyboard and mouse with SOFTmax PRO, you may generate and save different plate formats. Refer to reference III.D. regarding specific methods of programming.

E. Data Analysis

1. The raw data is the output containing the absorbencies of those wells used. Unused wells need not be considered. Print the raw data report immediately after data generation and prior to manipulating any data (e.g., masking of wells). Print out any changes made in the raw data report with the correction made IAW GLP regulations (see reference III.B.). Initial and date these records at the time of printout.

2. A standard curve may be generated by inputting known values for designated sample wells. Refer to the SOFTmax PRO instruction manuals for specific instructions regarding standards (see reference III.D.). Place the raw data in the study file.

3. Perform necessary editing of reports (as described in the software and equipment manuals) after printing out the original raw data.

F. Maintenance

1. Maintain an equipment logbook and document the use and maintenance of the microplate reader. Replace the lamp only when necessary and note changes in the logbook.

G. Troubleshooting: There is a troubleshooting guide located in reference III.D.

H. Quality Control

1. For each assay read on the microplate reader, the operator will perform the appropriate controls (e.g., blanks, positive and negative controls, standard curve).

2. Calibration – Twice a year, perform test Plans 2 through 5 of the Validation Protocol for this instrument, when in use, and label it with its calibration status.

APPENDIX B

MREF *In Vitro* Method No. 53-01

METHOD FOR THE ELECTROPHORETIC MOBILITY SHIFT ASSAY

A. Statement of Work: This method is used for the extraction and quantification of nuclear proteins from tissue samples for use in the Electrophoretic Mobility Shift Assay (EMSA). EMSAs are designed to detect the presence of DNA binding proteins by their ability to retard the migration of DNA fragments as they are electrophoresed. The assay is performed by incubating protein samples with a ^{32}P end-labeled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a non-denaturing polyacrylamide gel. The method for protein extraction from tissue samples is based on that of Blackwell et. al. (1997).

B. Materials: The following materials or their equivalents are used:

1. Purchased from Worthington Biochemical Co., Freehold, NJ:
    a. G-25 Sephadex Spin Columns, Cat. No. LS004404
2. Purchased from NEN Life Science Products, Boston, MA:
    a. EasyTides Adenosine 5'-triphosphate, Cat. No. BLU502A
3. Purchased from BioRad, Hercules, CA:
    a. Cellophane Membrane Backing, Cat. No. 165-0963
    b. Sequencing Filter Paper, Cat. No. 165-0959
    c. 10X Tris-Borate-EDTA, Cat. No. 161-0733
    d. Bromophenol Blue, Cat. No. 161-0404
    e. Ready Gel, 5% TBE, Cat. No. 161-1127
    f. Power Pack Model 200/2.0, Cat. No. 165-5052
    g. Model 583 Gel Dryer, Cat. No. 165-1745
4. Purchased from USA Scientific, Ocala FL:
    a. Tiplet Rad-Tip Box, Cat. No. 3056-6000

b. Beta Rack, Cat. No. 3040-0000

c. Angled Shield, Cat. No. 3051-0000

5. Purchased from Sigma Chemical Co., St. Louis, MO:

a. Exposure Cassette, Cat. No. E-9385

b. IGEPAL CA-630, Cat. No. I-8896

c. HEPES, Cat. No. H-0763

d. Magnesium Chloride, Cat. No. M-1028

e. Glycerol, Cat. No. G-5516

f. Potassium Chloride, Cat. No. P-9541

g. Tris-HCL, Cat No. T-7149

h. Trizma Base, Cat. No. T-8524

i. Leupeptin, Cat. No. L-9783

j. Aprotinin, Cat. No. 62070

k. Phenylmethylsulfonyl Fluoride, Cat. No. P-7626

l. Sterile Distilled Water, Cat. No. W-4502

m. Sodium Hydroxide, Cat. No. S-8526

n. X-OMAT AR Film (Kodak), Cat. No. 165-1454

o. Intensifying Screen (Kodak), Cat. No. Z35,699-9

p. Pepstatin A (Fluka), Cat. No. 77170

6. Purchased from Promega Corp., Madison, WI:

a. Sodium Chloride, Cat. No. V422A

b. EDTA, Cat. No. V423A

c. Dithiothreitol, Cat. No. V315A

d. T4 Polynucleotide Kinase, Cat. No. M4101

e. NF-kappaB Oligonucleotide, Cat. No. E3291

f. HeLa Nuclear Extract, Cat. No. E3521

g. Gel Shift Assay Core System, Cat. No. E3050

h. NF-kappaB (p50, human), Cat. No. E3770

7. Purchased from Rainin, Woburn, MA:

   a. FinePoint Aerosol Resistant Tips, Cat. No. RT-10GF, HR-200F

8. Purchased from Pharmacia, Iscataway, NJ:

   a. Poly (dI-dC), Cat. No. 27-7880

9. Purchased from Pierce Chemical Co., Rockford, IL:

   a. BSA Protein Standards, Cat. No. 23209

   b. BCA Protein Assay Kit, Cat. No. 23223, 23224

10. Purchased from Daigger, Lincolnshire, IL:

    a. Dounce Tissue Grinder, Cat. No. NX24835B

11. Purchased from Fisher Scientific, Pittsburgh, PA:

    a. Parafilm M Laboratory Wrapping Film, Cat. No. 13-374-5

C. Equipment: Freezer, refrigerator, label tape, first-aid kit, weighing paper, wiping tissues, polypropylene snap-cap test tubes, Eppendorf microcentrifuge tubes, laboratory coat, safety shoes, safety glasses, disposable nitrile gloves, Eppendorf pipettors with pipette tips, vortex mixer, picofuge, Mettler AE 100 balance with calibration standards, mortar and pestle, ice buckets with ice, electrophoresis power supply, vertical gel electrophoresis apparatus, microplate reader, pH meter with calibration standards, metal spatulas, plastic weigh boats, 10 mL polystyrene pipette tips with pipettor, polyallomar tubes,

liquid nitrogen, dewar, cryovials, cryo-safe gloves, microcentrifuge tube racks, safe-light, X-ray film developer, Harvard balance, Beckman Avanti centrifuge with F1010 rotor, Eppendorf 5417R microcentrifuge/rotor, dry heat block, incubator, 540 nm filter, Beckman LS 3801 Liquid Scintillation Counter, scintillation vials, minigel apparatus, vacuum dryer apparatus.

D. Procedures:

1. Work Area and Instrument Set-Up: The work area for sample-handling is a clean laboratory benchtop. The centrifuges are allowed to equilibrate to approximately 4C.

2. Reagent Preparation:

   a. Homogenization Buffer A: In a clean 100 mL glass bottle combine approximately 0.6 mL of IGEPAL CA-630, 3.0 mL of 5M NaCl stock solution, 0.260 g of HEPES, and 0.200 mL of 0.5M EDTA stock solution. Raise the volume to approximately 100 mL with sterile distilled water. Adjust the pH of the solution to approximately 7.9. The shelf life of this preparation is approximately two weeks when stored at approximately 4C. Just before use in tissue sample homogenization add approximately 5 µL of 0.1M PMSF per mL of Buffer A and 1 µL of 1M DTT per mL of Buffer A.

   b. Homogenization Buffer B: In a clean 100 mL glass bottle combine approximately 25 mL of glycerol, 0.520 g of HEPES, 8404 µL of 5M NaCl stock solution, 120 µL of 1M $MgCl_2$ stock solution and 40 µL of 0.5M EDTA stock solution. Raise the volume to approximately 100 mL with sterile distilled water. Adjust the pH of the solution to approximately 7.9. The shelf life of this preparation is approximately two weeks when stored at approximately 4C. Just before use in tissue homogenization add approximately 5 µL of 0.1M PMSF per mL of Buffer B, 1 µL of 1M DTT per mL of Buffer B, 1 µL of 5 µg/µL pepstatin A per mL of Buffer B, 1 µL of 5 µg/µL leupeptin per mL of Buffer B, and 1 µL of 5 µg/µL aprotinin per mL of Buffer B.

   c. Homogenization Buffer C: In a clean 100 mL glass bottle combine approximately 40 mL of 20mM HEPES stock solution, 10 mL of glycerol, 20 µL of 0.5M EDTA stock solution, and 186.5 mg of KCL. Adjust the pH of the solution to approximately 7.9. The shelf life of this preparation is approximately two weeks when stored at approximately 4C. Just before use in tissue homogenization add approximately 1 µL of 1M DTT stock solution per mL of Buffer C,

approximately 5 µL of 0.1M PMSF per mL of Buffer C 2
approximately 1 uL of 5 ug/uL pepstatin A per mL of Buffer C, and
approximately 1 uL of 5 mg/uL leupeptin per mL of Buffer C.

d. 20 mM HEPES: In a clean 100 mL glass bottle combine approximately 521 mg of HEPES with approximately 100 mL of sterile distilled water. Adjust the pH of the solution to approximately 7.9. The shelf life of this preparation is approximately two weeks when stored at approximately 4C.

e. Poly (dI-dC) ds Copolymer: First prepare the reconstitution buffer by combining approximately 0.060g of Trizma base (FW 121.1) with approximately 100 µL of 0.5M EDTA and approximately 1 mL of 5M NaCl in a 50 mL conical tube. Raise the volume to approximately 50 mL with sterile distilled water. Adjust the pH of the solution to approximately 7.5. The shelf life of this preparation is approximately one year when stored at approximately 4C. The number of units per A_{260} of the copolymer determines the volume of reconstitution buffer needed. Use the formula (10U / A_{260} units/mg) x 1000 = the number of microliters (µL) to add to the lyophilized powder. Aliquot approximately 25 µL volumes into microcentrifuge tubes. The shelf life is approximately one year when stored at approximately –20C.

f. 0.1M PMSF: Dissolve approximately 0.085g of crystalline PMSF in approximately 5 mL of absolute ethanol. Note: **phenylmethylsulfonyl fluoride is a toxic compound and is an inhibitor of human acetylcholinesterase and should be handled with extreme care.** Use a balance located in a ventilated hood to address safety concerns. The shelf life of this preparation is approximately two years when stored at approximately –20C.

g. 5X EMSA Incubation Buffer: In a 100 mL glass bottle combine approximately 0.788g of Tris-HCl, 5 mL of 5M stock solution of NaCl, 500 µL of 1M stock solution of $MgCl_2$, 250 µL of 1M stock solution of DTT, 500 µL of 0.5M stock solution of EDTA, and 20 mL of glycerol. Raise the volume to approximately 100 mL with sterile distilled water. Adjust the pH to approximately 7.5. Sterile filter and aliquot approximately 1 mL volumes into microcentrifuge tubes. The shelf life of this preparation is approximately one year when stored at approximately –20C.

h. BCA Assay Reagent: In a 15 mL conical combine approximately 9.8 mL of Pierce Reagent A with 200 µL of Pierce Reagent B. Vortex the

solution. The shelf life of this preparation is approximately one day when stored at approximately 24C.

i. Protein Assay Standards: Add 1 mL of sterile distilled water to a snap-cap polypropylene tube labeled as 0 mg/mL. Add 1950 µL of sterile water and 50 µL of BSA Standard Stock Solution (2 mg/mL) to a tube labeled 50 µg/mL. Add 1900 µL of sterile water and 100 µL of BSA Standard Stock Solution (2 mg/mL) to a tube labeled 100 µg/mL. Add 900 µL of sterile water and 100 µL of BSA to a tube labeled 200 µg/mL. Add 400 µL of sterile water and 100 µL of BSA to a tube labeled 400 µg/mL. Add 233 µL of sterile water and 100 µL of BSA to a tube labeled 600 µg/mL. Add 150 µL of sterile water and 100 µL of BSA to a tube labeled 800 µg/mL. Add 100 µL of sterile water and 100 µL of BSA to a tube labeled 1000 µg/mL.

j. 0.5X TBE Electrophoresis Buffer: Dilute approximately 25 mL of 10X TBE Stock in approximately 500 mL of sterile distilled water. The shelf life of this preparation is approximately one year when stored at approximately 4C.

k. Bromophenol Blue Loading Dye: Combine approximately 25 mL of glycerol, 0.060g of Tris-HCl, and 0.019g of EDTA in a 50 mL conical tube. Mix by inversion. Adjust the pH of the solution to approximately 8.0. Add just enough bromophenol blue powder to give the solution a deep blue color. The shelf life of this preparation is approximately two years when stored at approximately 24C.

3. Preparation of Tissue Samples For Protein Isolation: Previously harvested tissue samples are stored in cryo-vials at below approximately –70C. When removed from the freezer for processing, the tissue samples are maintained in liquid nitrogen.

a. Using a calibrated Mettler analytical balance, obtain the weight of the tissue sample and record the data on Form No. MREF *In Vitro*-103 under "Notes".

b. Place the sample into a liquid nitrogen-cooled mortar. Add enough liquid nitrogen to submerse the sample. Reduce the sample to a fine powder with a liquid nitrogen-cooled pestle.

c. Use a cooled spatula to transfer the powder into a 15 mL Dounce homogenizer that contains approximately 4 mL of cold Tissue Homogenization Buffer A (with appropriate protease inhibitors).

d. Incubate on ice for approximately 15 minutes. Additional samples may be homogenized during this incubation.

e. "Dounce" the sample using approximately three strokes with the loose fitting plunger followed by five strokes with the tight fitting plunger.

f. Transfer the tissue suspension to a 10 mL Beckman polyallomer tube. Incubate the tissue suspension on ice while additional samples are being processed.

g. Centrifuge the sample(s) at approximately 3068 rpm (800 x g) in the Beckman Avanti, using the F1010 rotor, at approximately 4C for approximately 30 seconds.

h. Transfer approximately 3 mL of the supernatant(s) into clean Beckman polyallomer tubes. Centrifuge the supernatant(s) at approximately 6509 rpm (3600 x g) in the Beckman Avanti, using the F1010 rotor, at approximately 4C for approximately 15 minutes.

i. Decant the supernatant(s) to waste, blotting dry the lip of each tube. Suspend the nuclear pellet(s) in approximately 50 µL of Homogenization Buffer B (with the appropriate protease inhibitors added). Transfer the suspension(s) to microcentrifuge tubes.

j. Incubate the samples on ice for approximately 30 minutes with intermittent vortexing.

k. Microfuge the sample(s) using a Brinkman Eppendorf 5417R centrifuge at approximately 4C and 13000 rpm (17,900 x g) for approximately 5 minutes.

l. Aliquot the supernatant(s) into labeled microcentrifuge tubes which contain approximately 150 µL of Homogenization Buffer C (with the appropriate protease inhibitors added).

m. Store the nuclear protein sample(s) in labeled microcentrifuge tubes at –70C.

4. Protein Determinations:

a. Prepare the Protein Assay Standards and the BCA Reagent as stated in Reagent Preparation (Section D. 2., h. and i.).

b. Thaw the protein samples and keep on ice.

c. Dilute the protein samples 1:10 by withdrawing approximately 5 µL from each sample and mixing with approximately 45 µL of sterile distilled water.

d. Pipet approximately 20 µL aliquots of the appropriate BSA standard or diluted protein sample into the wells of a 96-well microplate.

e. Add approximately 200 µL of BCA Assay Reagent to each protein sample or BSA standard on the microplate. Additionally, pipet a duplicate sample of BCA Reagent to be used to assess reagent background.

f. Cover the microplate with parafilm and incubate at approximately 37C for approximately 30 minutes.

g. Return the remaining volume of the protein samples to storage at below –70C.

h. Use a microplate reader to determine the absorbance at a wavelength of approximately 540 nm. Record the data using the Molecular Devices SOFTmax Pro software, export into an Excel spreadsheet, and print for the study notebook. The results, given in OD units, can be converted to µg/mL concentration values via the OD readings that result from the known values of the BSA standards.

i. Calculations based on data from the microplate readings are performed to determine the volume of protein sample required for an appropriate load with which to perform the EMSA reactions. Typically, between five and twenty µL of protein is sufficient per EMSA assay, with the amount used being subject to the investigator's discretion.

5. Preparation of the NF*k*B ds DNA Probe:

a. Equilibrate a dry block heater to approximately 37C.

b. Add the approximate volumes of the following components to a microcentrifuge tube: 2 µL of NF-kappa B Oligonucleotide (1.75 pmol/µL), 1 µL of 10X Forward Reaction Kinase Buffer, and 4 µL of sterile distilled water.

c. Don two pairs of nitrile gloves, the required radiation safety monitoring devices, and stand behind the acrylic shielding while adding approximately 2 µL of [gamma ^{32}P]ATP (10 uCi/µL) to the microcentrifuge tube that contains the previously added reaction components.

d. Add approximately 1 µL of T4 polynucleotide kinase enzyme to start the reaction. Pipet to mix the components, picofuge briefly, and set the microcentrifuge tube in the equilibrated dry heat block.

e. Allow the reaction to proceed for approximately 10 minutes at approximately 37C.

f. Stop the reaction by adding approximately 1 µL of 0.5M EDTA.

g. Dilute the reaction by adding approximately 89 µL of TE buffer.

h. Prepare a G-25 sepharose micro-column by centrifuging it at approximately 13,000 rpm (17,900 x g) for approximately three minutes at room temperature.

i. Take an approximately 1 µL pre-count sample of the reaction that labeled the probe for quantitation in the Beckmen LS 3801 scintillation counter.

j. Add the diluted probe reaction to the prepared G-25 column.

k. Centrifuge the probe reaction through the G-25 column at approximately 13,000 rpm (17,9000 x g) for approximately two minutes at room temperature to purify the reaction.

l. Take an approximately 1 µL post-count sample of the purified reaction for scintillation counting in the Beckman LS 3801.

m. The pre and post-count samples, when read by the LS 3801, will provide the activity of the purified end-labeled probe in counts per minute (cpm) of radioactivity. Calculate the amount of diluted probe that should be added to each experimental protein sample to be assayed (see below) so that approximately 30,000 cpm are added to each EMSA assay.

n. The purified probe can now be used in an EMSA reaction. The shelf life of the end-labeled probe is approximately one week when stored at approximately –20C.

6. EMSA Reactions:
   a. Thaw the end-labeled probe behind the protective acrylic shielding in the hood.

   b. Thaw the previously harvested protein samples on ice. Also set on ice aliquots of poly (dI-dC), 5X EMSA incubation buffer, HeLa control protein, competitor oligos (SP-1 and/or AP-2), and sterile distilled water.

   c. Following the experimental outline on Form No. MREF *In vitro*-095 add the appropriate quantities of experimental protein samples (as indicated by the results of the protein determination), poly (dI-dC), 5X EMSA incubation buffer, HeLa control protein, competitor oligos (SP-1 and/or AP-2), and sterile distilled water to labeled microcentrifuge tubes on ice.

   d. Allow the reactions to remain on ice for approximately 15 minutes.

   e. Behind the protective acrylic shielding add the amount of radioactive probe per sample that is indicated on Form No. MREF *In vitro*-095.

   f. Allow the tubes to incubate at room temperature for approximately 20 minutes.

   g. Add the amount of 10X bromophenol blue gel loading dye that is indicated on Form No. MREF *In vitro*-095.

   h. Working carefully behind the protective acrylic shielding, add sufficient 0.5X TBE electrophoresis buffer to the chamber, load the samples into the wells of a 5% TBE BioRad Ready Gel, and start the electrophoresis at approximately 140 volts, constant voltage.

   i. Electrophorese the samples until the bromophenol blue dye front reaches approximately 4/5th of the distance down the vertical face of the gel.

   j. Stop the electrophoresis. Take apart the electrophoresis apparatus to release the gel cassette. Using a razor blade, cut along the margins as

indicated on the gel cassette. Use the razor blade to separate the plates of the cassette, allowing the acrylamide gel to adhere to only one plate.

k. Transfer the gel to an appropriately sized piece of filter paper, being sure to notch the bottom left corner of the gel in order to mark the first lane, which corresponds to the first experimental sample reaction. Cover the gel with a piece of cellulose film.

l. Vacuum-dry the gel at approximately 80C for approximately 1.5 hours.

m. Expose the dried gel to X-ray film at approximately –20C in a cassette that has an intensifying screen. Develop the film after an appropriate exposure time. Additional exposures may be acquired if deemed necessary.

E. References:

1. Blackwell TS, Blackwell TR, and JW Christman. 1997. *The Journal of Immunology*. Impaired Activation of Nuclear Factor-*k*B in Endotoxin-Tolerant Rats Is Associated with Down-Regulation of Chemokine Gene Expression and Inhibition of Neutrophilic Lung Inflammation.

2. Promega Corporation Technical Bulletin No. 110. 1996. Gel Shift Assay Systems.